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Suprageneric Phylogeny of Japanese Cyperaceae Based on DNA Sequences from Chloroplast *ndhF* and 5.8S Nuclear Ribosomal DNA

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The phylogeny of Japanese Cyperaceae was investigated using chloroplast *ndhF* sequence data from 37 species in 27 genera. Our results show some congruence with the classification of Goetghebeur (1998). The 5.8S rDNA was analyzed for 37 species in 24 genera. Three base substitutions and 1-bp deletion were observed. A 3-bp indel (CAT) was found in many genera of Cyperaceae. Our results indicate that these mutations of 5.8S rDNA occurred in the ancestor of the family. The monophyly of subfamilies *Mapanioideae*, *Sclerioideae*, and *Caricoideae* is strongly supported. However, the monophyly of subfamily *Cyperoideae* is not supported because subfamily *Caricoideae* is nested within it. The monophyly of tribes *Cariceae*, *Abildgaardieae*, *Eleocharideae*, and *Cypereae* is strongly supported, but *Schoeneae*, *Scirpeae*, and *Fuireneae* are polyphyletic. Two groups were distinguished in the *ndhF* tree: (1) *Dulichieae* to *Cariceae*, and (2) *Cypereae* to *Abildgaardieae*.

Key words: 3-bp indels, 5.8S rDNA, Cyperaceae, *ndhF*, subfamilial classification, tribal classification

The Cyperaceae are the third largest family of the Monocotyledons, comprising 104 genera and over 5000 species. They have a cosmopolitan distribution, occurring in a variety of habitats ranging from the humid tropics to cold temperate regions (Goetghebeur 1998). In Japan, Ohwi (1944) recognized 20 genera and over 450 species. Koyama (1961), following the system of Ohwi (1944), recognized four subfamilies and six tribes of Japanese Cyperaceae based mainly on floral morphology (Fig. 1). Ohwi (1944) and Koyama (1961) both hypothesized that the unisexual flowers of Cyperaceae evolved from bisexual flowers. The sex and morphology of the flower are therefore important characters for the subfamilial and tribal classification of the family.

The more recent systems of Bruhl (1995) and Goetghebeur (1998) were based on morphological, anatomical, embryological, phytochemical, and physiological analyses. Bruhl (1995) recognized two subfamilies and 12 tribes of Cyperaceae (Fig. 1). His system was based on cladistic and phenetic analyses of DELTA (Description Language for Taxonomy) datasets (Dallwitz *et al.* 1993, Partridge *et al.* 1993). He classified four tribes with bisexual flowers in the subfamily *Cyperoideae*, and placed the other tribes into subfamily *Caricoideae* (Fig. 1). Goetghebeur (1998) recognized four subfamilies and 14 tribes, mainly based on floral and embryonic characters (Fig. 1). He classified nine tribes with bisexual flowers into two subfamilies *Cyperoideae* and *Mapanioideae*, and classified five tribes with unisexual flowers into two subfamilies *Caricoideae* and *Sclerioideae*.

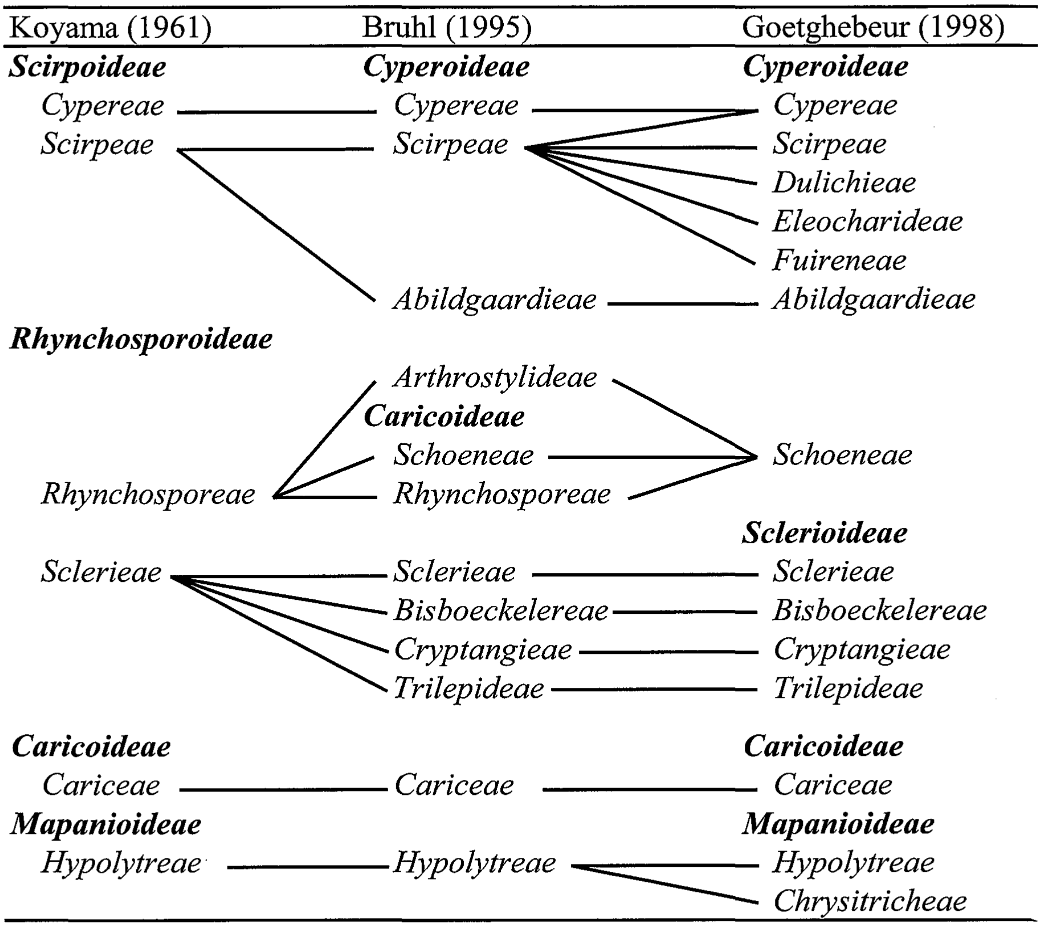

FIG. 1. Comparison of subfamilial and tribal classifications of Cyperaceae.

Molecular data are providing a better understanding of within- and between- tribal relationships in Cyperaceae (Simpson *et al.* 2003). Muasya *et al.* (1998) provided the first molecular phylogenetic tree of the family using *rbcL* sequence data from 80 species in 40 genera. Muasya *et al.* (2000) revised the phylogeny of Cyperaceae using *rbcL* sequence and morphological data from 58 species in 54 genera from Africa, Australia, North America, Europe, Southeast Asia, and South America. The molecular phylogenetic trees of Muasya *et al.* (1998, 2000) do not support the subfamilial classifications of Bruhl (1995) and Goetghebeur (1998). Based on *trnL-F* region and *rps16* gene (Simpson *et al.* 2003), the phylogeny at the subfamilial level of Cyperaceae indicates that subfamily *Mapanioideae* is sister to the rest of the family. At present, only three molecular phylogenetic analyses have been conducted at the suprageneric level of Cyperaceae, none of which include the Japanese species (Muasya *et al.* 1998, 2000, Simpson *et al.* 2003).

The *rbcL* data of Muasya *et al.* (1998, 2000) was insufficient for clarifying the relationships among the tribes of Cyperaceae. Other DNA data are necessary to provide a more detailed view of the relationships. Chloroplast *ndhF* has a higher rate of base substitution than *rbcL*, making it suitable for studies at the generic level and above (Olmstead & Sweere 1994, Clark *et al.* 1995, Bohs & Olmstead 1997, Terry *et al.* 1997), therefore increasing the potential to better resolve suprageneric relationships in the family.

Starr *et al.* (1999) reported a 3-bp insertion (CAT) near the 3′ end of 5.8S ribosomal DNA (rDNA) in *Carex* and *Kobresia* (position 155-157). Five types of 3-bp indels (CAC, CAT, CTC, CTT, and 3-bp deletion) were found in *Eleocharis* (Yano *et al.* 2004). They proposed that 3-bp indels are useful for delimiting clades within a genus or tribe. The mutations in the 3-bp indel, however, were investigated only in these three genera of Cyperaceae.

The goal of this study is to clarify the subfamilial and tribal relationships in Japanese Cyperaceae using chloroplast *ndhF* and nuclear 5.8S rDNA sequence data, and to identify the 3-bp indels of 5.8S rDNA in the Japanese members of the family.

Materials and Methods

The chloroplast *ndhF* gene from 37 species in 27 genera of Cyperaceae was sequenced and sequence data for three species were obtained from DDBJ/EMBL/GenBank (Table 1). *Juncus decipiens* (Juncaceae) and *Oryza sativa* (Poaceae) were used as outgroups. Their *ndhF* sequences were determined in this study or were obtained from DDBJ/EMBL/GenBank. The 5.8S rDNA region was analyzed for 37 species in 24 genera, using nine published sequences from Cyperaceae, three from Juncaceae and one from *Oryza sativa* (Poaceae) (Takaiwa *et al.* 1985, Roalson & Friar 2000, Yano *et al.* 2004).

DNA Extraction, PCR Amplification and DNA Sequencing

Total DNA was extracted from 0.1g fresh-frozen material or 0.03 g dried (silica gel dried or herbarium specimens) samples using the Nucleon Phytopure plant and fungal DNA extraction kit (Amersham Inc.). Total DNA preparations were purified by ethanol precipitation. The PCR reaction mixture contained 50 ng of genome DNA, 0.2 μM of 5′ and 3′ end primers, 25 μL of Premix Taq (TaKaRa Bio Inc.), and 5% Dimethyl sulfoxide in a total volume of 50 μL. Reactions were performed using a DNA thermal cycler GeneAmp PCR System 2400 (Perkin Elmer Inc.). We designed four new primers: *ndhF*-3F (5′-GGAACAGACATAT-CAATATG-3′), *ndhF*-922F (5′-GAGCCACTT-TAGCTCTTGCT-3′), *ndhF*-1163R (5′-TAAACC ACCCATAAGAACCA-3′) and *ndhF*-2016R (5′-TAACCACGATTATATGACCA-3′). Two primer sets were used for PCR amplification of the *ndhF* gene segments. One primer set was *ndhF*-3F and *ndhF*-1163R; the other set was *ndhF*-922F and *ndhF*-2016R. The reaction profile for *ndhF* amplification involved: denaturation at 94° C for 5 min; 40 cycles at 92°C for 1 min, 48°C for 1 min and 72°C for 2 min; and a single final extension at 72° C for 7 min. The nrDNA ITS fragments were amplified using two primers, ITSL (5′-TCGTAACAA-GGTTTCCGTAGGTG-3′; Hsiao *et al.* 1994) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990), following the protocol of Hsiao *et al.* (1994).

The PCR products were electrophoresed on 1.4% agarose gels to confirm a single band, and purified using the QIAquick PCR Purification Kit (QIAGEN Inc.). Direct DNA sequence analysis of the PCR products was performed using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Inc.) and analyzed using automated capillary electrophoresis ABI PRISM 310 (Applied Biosystems Inc.). The sequences obtained were submitted to the DDBJ/EMBL/GenBank databases.

Sequence Analysis

Data set - The species treated in this study are indicated in Table 1, along with their DDBJ/EMBL/GenBank accession numbers. For the analyses, we used two data sets. To estimate the phylogenetic relationships among the genera of Cyperaceae data set I (*ndhF* sequences) included 39 species. The matrix of data set II (5.8S sequences), including 41 species, was used to determine the distribution of the unique 3-bp insertion in

TABLE 1. Taxa used in this study. Subfamilial and tribal classification of Cyperaceae according to Goetghebeur (1998). Vouchers are deposited in the herbarium of Okayama University of Science, Okayama (OKAY). Asterisks show literature citation for previously published sequences.

Taxon	Locality and voucher	Accession number	
		ndhF	ITS
Cyperaceae			
Caricoideae			
Cariceae			
Carex albata Boott ex Franch. et Sav.	Japan, Hokkaido Pref., Kyowa; *Hoshino et al. 17400*	AB261626	AB261661
Kobresia nepalensis (Nees) Kük.	Nepal; *Hoshino et al. 9660052*	AB261627	AB261662
Uncinia phleoides (Cav.) Pers.	Yen & Olmstead (2000)*	AF163467	
Cyperoideae			
Abildgaardieae			
Bulbostylis barbata (Rottb.) Kunth	Japan, Okayama Pref., Okayama; *Hoshino et al. 13779*	AB261628	AB180718
B. densa (Wall.) Hand. -Mazz.	Japan, Okayama Pref., Soujya; *Hirahara et al. 19651*	AB261629	AB261663
Fimbristylis velata R. Br.	Japan, Okayama Pref., Tamano; *Hoshino et al. 17692*	AB261630	AB261664
Cypereae			
Cyperus cyperoides (L.) O. Kuntze	Japan, Okayama Pref., Yakake; *Morimoto 17532*	AB261631	AB261665
C. iria L.	Japan, Okayama Pref., Okayama; *Yano 18125*	AB261632	AB261666
C odoratus L.	Japan, Okayama Pref., Okayama; *Katayama 19301*	AB261633	AB261667
Isolepis crassiuscula Hook. f.	Japan, Fukushima Pref., Shirakawa; *Hirahara & Hoshino 19165*	AB261634	AB261668
Kyllinga brevifolia Rottb. subsp. *brevifolia*	Japan, Okayama Pref., Tamano; *Hirahara & Yano 18135*	AB261635	AB261669
Lipocarpha microcephala (R. Br.) Kunth	Japan, Okayama Pref., Sojya; *Katayama 17518*	AB261636	AB261670
Pycreus sanguinolentus (Vahl) Nees	Japan, Okayama Pref., Okayama; *Komagine & Masyo 17655*	AB261637	AB261671
Dulichieae			
Dulichium arundinaceum (L.) Britt.	Yen & Olmstead (2000)*	AF163472	
Eleocharideae			
Eleocharis acicularis (L.) Roem. et Schult. f. *longiseta* (Svenson) T. Koyama	Japan, Nagano Pref., Karuizawa; *Arai & Hirahara 18168*	AB261638	AB180693
E. congesta D. Don var. *japonica* (Miq.) T. Koyama	Yano *et al.* (2004)*		AB180697
E. interstincta (Vahl) Roem. et Schult.	Roalson & Friar (2000)*		AF190611
E. kuroguwai Ohwi	Japan, Okayama Pref., Satosyo; *Katayama 17643*	AB261639	AB180704
E. mamillata Lindb. f.	Yano *et al.* (2004)*		AB180705
Fuireneae			
Actinoscirpus grossus (L. f.) Goetgh. et D. A. Simpson	Japan, Tokyo Pref., Hahajima; *Katsuyama et al. 19915*	AB261640	AB261672
Bolboschoenus yagara (Ohwi) Y. C. Yang et M. Zhan	Japan, Aomori Pref., Misawa; *Hoshino et al. 13020*	AB261641	AB261673
Fuirena umbellata Rottb.	Japan, Okinawa Pref., Ishigaki; *Hirahara & Yano 19320*	AB261642	AB261674
Schoenoplectus hotarui (Ohwi) Holub	Japan, Hokkaido Pref., Tomakomai; *Hirahara et al. 19269*	AB261643	AB206259
Schoeneae			
Cladium jamaicense Crantz subsp. *chinense* (Nees) T. Koyama	Japan, Okayama Pref., Ushimado; *Izumi et al. 16881*	AB261644	AB261675
Gahnia aspera (R. Br.) Spreng.	Japan, Tokyo Pref., Chichijima; *Katsuyama et al. 19924*	AB261645	AB261676
G. tristis Nees	Japan, Okinawa Pref., Iriomote; *Hirahara 20361*	AB261646	AB261677
Machaerina glomerata (Gaudich.) T. Koyama	Japan, Tokyo Pref., Chichijima; *Katsuyama et al. 19874*	AB261647	AB261678
M. rubiginosa (Sol. ex G. Forst.) T. Koyama	Japan, Ehime Pref., Imabari; *Hoshino et al. 19254*	AB261648	AB261679
Rhynchospora chinensis Nees et Meyen	Japan, Okayama Pref., Tamano; *Hoshino et al. 17693*	AB261649	AB261680
R. rubra (Lour.) Makino	Japan, Okayama Pref., Ushimado; *Hoshino 18970*	AB261650	AB261681
Schoenus apogon Roem. et Schult.	Japan, Okayama Pref., Ushimado; *Hoshino 18971*	AB261651	AB261682
S. brevifolius R. Br.	Japan, Tokyo Pref., Chichijima; *Katsuyama et al. 19875*	AB261652	AB261683

TABLE 1. Continued.

Taxon	Locality and voucher	Accession number	
		ndhF	ITS
Cyperaceae			
Cyperoideae			
Scirpeae			
Eriophorum gracile K. Koch	Japan, Hokkaido Pref., Tomakomai; *Hoshino et al. 17382*	AB261653	AB261684
E. vaginatum L.	Japan, Iwate Pref., Takizawa; *Kameyama & Yano 18975*	AB261654	AB261685
Scirpus wichurae Böeck.	Japan, Okayama Pref., Sojya; *Katayama 17510*	AB261655	AB261686
Trichophorum alpinum (L.) Pers.	Japan, Hokkaido Pref., Nakagawa; *Sato 13260*	AB261656	AB206270
T. cespitosum (L.) Hartm.	Japan, Iwate Pref., Ashiro; *Katsuyama & Nakayama 20318*	AB261657	AB261687
Mapanioideae			
Hypolytreae			
Mapania paradoxa J. Raynal	Givnish *et al.* (2002)*	AY129256	
Sclerioideae			
Bisboeckelereae			
Diplacrum caricinum R. Br.	Japan, Okayama Pref., Sojya; *Katayama 19302*	AB261658	AB261688
Sclerieae			
Scleria parvula Steud.	Japan, Okayama Pref., Sojya; *Hoshino et al. 17555*	AB261659	AB261689
Outgroup			
Juncaceae			
Juncus decipiens (Buchenau) Nakai	Japan, Okayama Pref., Okayama; *Hirahara 17764*	AB261660	AB261690
Luzula capitata (Miq.) Miq. ex Kom.	Japan, Okayama Pref., Okayama; *Hirahara & Yano 17728*		AB261691
L. elegans Guthnick	Portugal; *Draper 17729*		AB261692
Poaceae			
Oryza sativa L.	Hiratsuka *et al.* (1989)*	X15901	
	Takaiwa *et al.* (1985)*		M16845

the 5.8S gene (Starr *et al.* 1999) and to assess its phylogenetic utility.

Alignment - DNA sequences were initially aligned using CLUSTAL W (Thompson *et al.* 1994) and then adjusted manually as necessary.

Phylogenetic analysis

Phylogenetic analyses were performed following the modified methods of Tsubota *et al.* (2002, 2003) and Yano & Hoshino (2005). Phylogenetic trees were constructed using both the maximum-parsimony (MP) (Fitch 1971), and maximum-likelihood (ML) (Felsenstein 1981) criteria. The trees obtained by the two methods were appraised by the log-likelihood measure with NucML in MOLPHY 2.3b3 (Adachi & Hasegawa 1996).

Parsimony analysis - In analysis with MEGA2 (Kumar *et al.* 2001), the MP trees were searched by close-neighbor-interchange (CNI) with search level 3, and the CNI search was repeated 10 times, starting with trees generated by 1000 replications with random addition sequences. In analysis by DNAPARS in PHYLIP 3.573c (Felsenstein 1980-2001), the MP trees were searched for the best tree using a heuristic search. The parsimony method used the default setting. The sequences order was non random. Input sequences were sequential. In analysis by DNAPENNY in PHYLIP 3.573c, the MP trees were searched using branch-and-bound exact searches. Again, the parsimony method used the default setting; the order was non random and input sequences were sequential. In analysis by PAUP*4.0b10 (Swofford 2003), the MP trees were searched using the parsimony Ratchet search strategy (PAUPRat; Nixon 1999) in twenty 200-iteration runs.

Maximum likelihood analysis - ML trees were constructed by NucML in MOLPHY ver. 2.3b3, DNAML in PHYLIP 3.573c, fastDNAml ver. 1.2.2 (Olsen *et al.* 1994). For the ML analyses, the HKY85 model (Hasegawa *et al.* 1985) was used as the estimate model. In analyses with NucML, the ML trees were searched with the local rearrangement method from the NJ tree by NucML and NJdist 1.2.5 (Adachi & Hasegawa 1996). In analyses by DNAML and fastDNAml, the ML trees were searched using global rearrangement options. The transition/transversion (TS/TV) parameter of DNAML and fastDNAml that best conforms to the data (4.644) was estimated based on calculations by MOLPHY.

Tree comparison -Tree comparisons with the ML criteria by NucML was used to evaluate the topology of trees obtained from Consel 0.1f (Shimodaira & Hasegawa 2001) and was also used to calculate the *p*-values of confidence for the bifurcating candidate topologies using test procedures.

Results

The ndhF *analysis*

The *ndhF* sequences of the 39 species, including two outgroups, ranged in length from 1893-2205 bp. Aligned sequences were 2248 bp, of which 1716 sites were used for the phylogenetic analyses. Twenty-two gaps, ranging from one to twelve sites long, were introduced in the *ndhF* sequences, but gaps were treated as missing data. The data matrix of *ndhF* contained 761 variable sites, of which 484 were potentially phylogenetically informative.

For phylogenetic analyses based on *ndhF* sequences, a total of 24 topologies were obtained from the MP and ML methods. The 18 non-overlapping topologies were obtained from the 24 topologies by CONSEL 0.1f. The best-supported ML tree was searched through the 18 non-overlapping topologies using a log-likelihood measure and *p*-values of AU, PP, and BP tests by CONSEL 0.1f. The best-supported ML tree with the highest likelihood value is shown in Fig. 2. The log-likelihood value for the ML tree was -14229.79 ± 353.5. AU, PP, and the BP tests values supported the ML tree more strongly than it supported the other trees.

Our data provide strong support for Cyperaceae being monophyletic (100% Local bootstrap probability: LBP, Fig. 2). Subfamily *Mapanioideae* is sister to the remaining species. Monophylies of subfamilies *Sclerioideae* and *Caricoideae* are supported, respectively (99 and 100% LBP), but the subfamily *Cyperoideae* is not a monophyletic group, because the clade of *Caricoideae* is nested within *Cyperoideae.* Tribe *Schoeneae* is a polyphyletic group including three lineages. The first lineage is the genus *Cladium* and is sister to the rest of subfamily *Cyperoideae* and subfamily *Caricoideae.* The second lineage comprises three genera *Gahnia*, *Schoenus* and *Machaerina*, and each genus is monophyletic. The last lineage is the genus *Rhynchospora*, which is sister to the clade of the remaining *Cyperoideae* and *Caricoideae.* The tribes *Cariceae*, *Scirpeae*, *Dulichieae*, *Abildgaardieae*, *Eleocharideae*, *Fuireneae*, and *Cypereae* form a monophyletic group. This group comprises two large clades, *Dulichieae* to *Cariceae* and *Cypereae* to *Abildgaardieae.* Most tribes of this clade are monophyletic, but *Fuireneae* and *Scirpeae* are polyphyletic.

5.8S analysis

The sequences of combined ITS 1 and 2 ranged in length from 209 to 409 bp, and sequences of 5.8S rDNA were 163 to 166 bp, including Juncaceae and Poaceae. Of them, only sequences of 5.8S rDNA were used, because the ITS regions were not strictly aligned. The data matrix of 5.8S (aligned length of 168 bp) contains 46 variable sites, of which 25 were potentially phylogenetically informative and five were indels (Table 2). The variability in 5.8S rDNA sequences was too low

TABLE 2. The 25 informative sites and five indels in 5.8S rDNA. Shaded boxes show the noticeable mutation.

	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0	0	0	0	1	1	1	2	3	3	6	9	0	0	2	2	3	3	3	3	3	3	3	3	5	5	5	6	6	6
	1	2	3	5	1	2	8	7	0	4	7	0	1	3	8	9	0	1	2	3	4	5	6	8	5	6	7	4	7	8
Cyperaceae																														
tribe *Cypereae*																														
Kyllinga brevifolia subsp. *brevifolia*	A	G	T	T	T	T	C	C	C	C	G	T	G	C	A	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Cyperus odoratus	A	T	T	C	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Pycreus sanguinolentus	A	T	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Cyperus iria	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	T	C	C	-	T	C	C	-	-	-	T	A	A
Cyperus cyperoides	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Lipocarpha microcephala	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Isolepis crassiuscula	A	T	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	T	C	C	-	T	C	C	-	-	-	T	A	A
tribe *Fuireneae*																														
Schoenoplectus hotarui	A	A	T	C	T	C	C	C	C	C	G	T	G	C	G	-	A	T	C	C	-	T	C	C	-	-	-	T	A	A
Actinoscirpus grossus	A	T	T	T	T	C	C	C	C	C	G	T	G	A	G	-	A	C	C	C	-	T	C	C	-	-	-	T	A	A
Fuirena umbellata	T	G	T	C	T	C	C	T	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	C	A	T	T	A	A
Bolboschoenus yagara	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
tribe *Eleocharideae*																														
Eleocharis mamillata	G	A	G	T	C	C	C	C	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	-	-	-	T	A	A
Eleocharis congesta var. *japonica*	G	A	G	T	C	C	C	C	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	C	T	T	T	A	A
Eleocharis interstincta	G	A	G	C	C	C	C	C	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	C	T	C	T	A	A
Eleocharis kuroguwai	G	A	G	T	C	C	C	C	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	C	A	C	T	A	A
Eleocharis acicularis f. *longiseta*	G	A	G	T	C	C	C	C	C	C	A	T	G	C	G	-	A	C	C	A	-	T	C	C	C	A	T	T	A	A
tribe *Abildgaardieae*																														
Bulbostylis densa	G	A	G	T	C	C	C	C	C	C	G	T	G	C	G	-	A	C	C	A	-	T	C	C	C	A	T	A	A	A
Bulbostylis barbata	G	A	G	T	C	C	C	C	C	C	G	T	G	C	G	-	A	C	C	A	-	T	C	C	C	A	T	A	A	A
Fimbristylis velata	G	A	G	T	C	C	C	C	C	C	G	T	G	C	G	-	A	T	C	A	-	T	C	T	C	A	T	A	A	A
tribe *Scirpeae*																														
Trichophorum alpinum	T	A	T	C	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Trichophorum cespitosum	T	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Scirpus wichurae	A	G	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Eriophorum vaginatum	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Eriophorum gracile	A	A	T	C	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
tribe *Cariceae*																														
Kobresia nepalensis	A	G	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Carex albata	A	A	T	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
tribe *Schoeneae*																														
Rhynchospora chinensis	A	A	G	T	T	C	C	C	C	C	G	T	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Rhynchospora rubra	A	A	G	T	T	C	C	T	C	C	G	C	G	C	G	-	A	C	C	C	-	T	C	C	C	A	T	T	A	A
Gahnia aspera	A	T	G	T	T	C	C	C	C	C	G	C	G	T	G	-	A	C	C	A	-	T	C	C	C	A	T	T	A	A
Gahnia tristis	A	T	G	T	T	C	C	C	C	C	G	C	G	C	G	-	A	C	C	A	-	T	C	C	C	A	T	T	A	A
Schoenus apogon	A	T	G	T	T	C	C	C	C	C	G	C	G	C	G	-	A	T	C	C	-	G	C	C	C	A	T	T	A	A
Schoenus brevifolius	A	C	G	T	T	C	C	C	C	C	G	T	G	C	G	-	A	T	C	C	-	G	C	C	-	-	-	T	A	A
Machaerina rubiginosa	T	T	G	T	T	C	C	C	C	C	G	C	G	C	G	-	A	C	C	C	-	G	C	C	-	-	-	T	A	A
Machaerina glomerata	T	C	G	T	T	C	C	C	C	C	G	C	G	C	G	-	A	C	C	T	-	G	C	C	-	-	-	T	T	A
Cladium jamaicense subsp. *chinense*	A	T	G	T	T	C	C	C	C	C	G	C	G	C	G	-	A	C	C	C	-	G	C	C	C	A	T	T	G	A
tribe *Sclerieae*																														
Scleria parvula	A	A	C	T	T	C	C	C	C	C	G	C	G	T	G	-	T	C	C	T	-	G	C	C	C	A	T	A	A	A
tribe *Bisboeckelereae*																														
Diplacrum caricinum	G	A	G	T	T	C	C	C	C	C	G	T	G	C	G	-	G	C	C	A	-	A	C	C	C	A	T	T	A	A
Juncaceae																														
Juncus decipiens	G	G	A	T	T	C	C	C	C	C	G	T	G	T	C	C	A	T	T	T	-	G	G	T	-	-	-	C	T	T
Luzula elegans	G	A	A	A	T	T	T	T	T	T	G	T	A	T	C	C	A	C	T	T	T	G	G	T	-	-	-	C	T	T
Luzula capitata	G	A	A	C	T	C	T	C	T	T	G	T	A	T	C	C	T	T	T	T	T	G	G	C	-	-	-	C	T	T
Poaceae																														
Oryza sativa	C	A	C	C	T	C	C	C	C	C	C	T	G	C	C	C	A	T	C	C	-	G	G	C	-	-	-	C	G	C

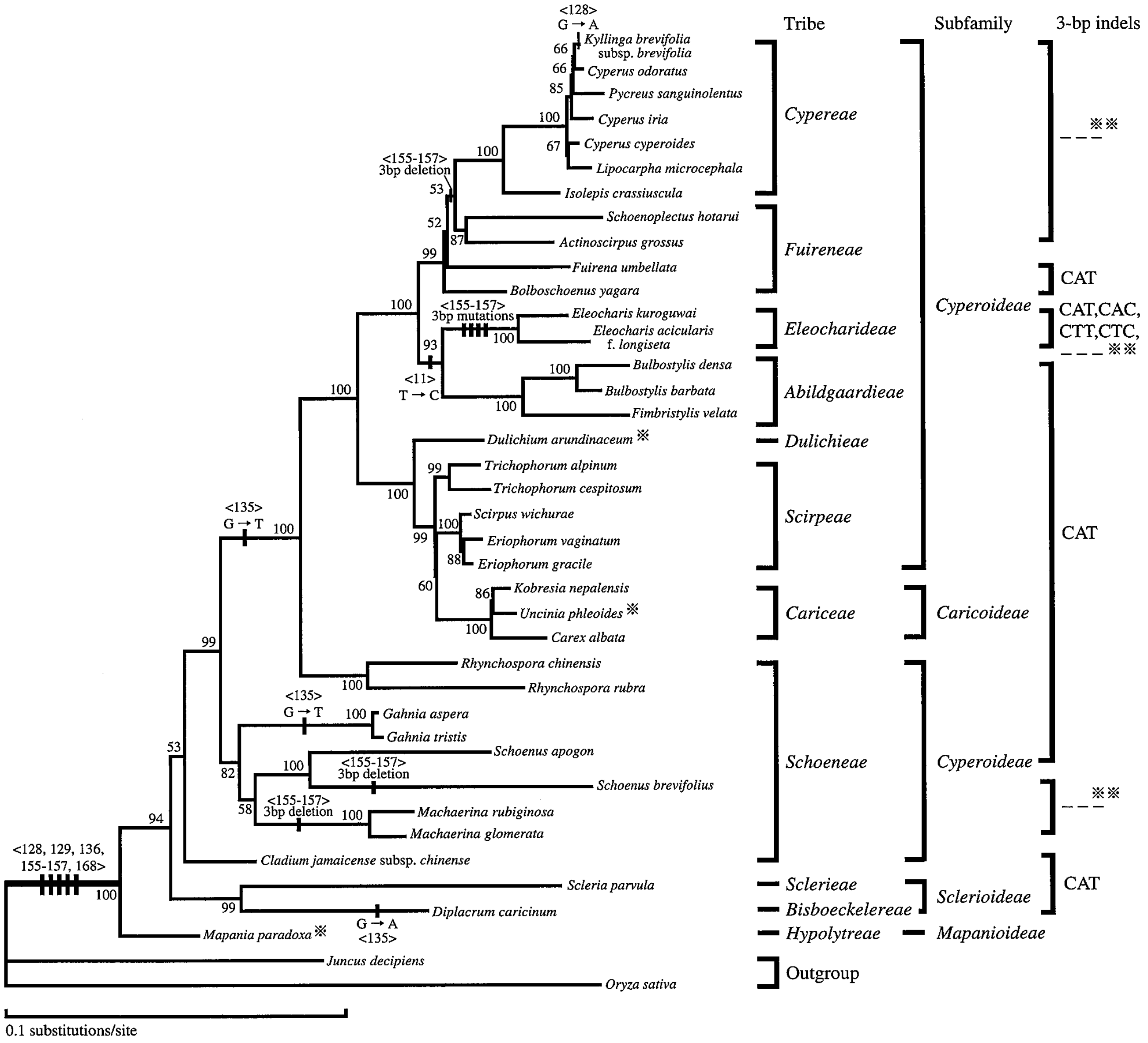

FIG. 2. The highest likelihood tree for the aligned 1716 bp of 39 *ndhF* gene sequences (HKY 85 model; $\alpha / \beta = 4.644$; $\ln L = -14229.79 \pm 353.5$ by NucML). The subfamilial and tribal classification by Goetghebeur (1998) is shown on the right. The root is arbitrarily placed on the branch leading to *Mapania paradoxa*. Local bootstrap probabilities (LBP; %) are shown near branches. ※ The taxa were not investigated of 5.8S rDNA. Three large capitals show a 3-bp indels of 5.8S rDNA (※※ 3-bp deletion). Bars across branches are supported by the noticeable mutations of 5.8S rDNA <the sequence number>.

to resolve the phylogenetic relationship at the supra-generic level. Eight sites were noticeable mutations arranged on the tree (Table 2, Fig. 2). Three base substitutions and 1-bp deletion (positions 128, 129, 136, and 168) were observed in Cyperaceae. Of the substitutions, for the single base substitution at position 128, the genus *Kyllinga* shows adenine (A), but the other genera show guanine (G). A single base substitution (position 11) is observed in tribes *Eleocharideae* and *Abildgaardieae*. A single base substitution (position 135) is shared in tribes *Schoeneae* (excluding *Machaerina*, *Schoenus*, and *Cladium*), *Cariceae, Scirpeae*, *Dulichieae*, *Eleocharideae*, *Abildgaardieae*, *Fuireneae*, and *Cypereae*. In the same position, another single substitution is observed in *Diplacrum* of tribe

Bisboeckelereae. A 3-bp insertion (CAT; position 155-157) is newly observed in the 12 genera of tribes other than tribe *Cariceae*. Eight genera of Cyperaceae do not have a 3-bp insertion. In addition, two types of 3-bp indels (CAT and 3-bp deletion) are found in *Schoenus*. Five types of 3-bp indels (CAC, CAT, CTC, CTT, and 3-bp deletion) were ascertained in *Eleocharis* (Table 2, Yano *et al.* 2004).

Discussion

The ndhF *phylogeny*

This study represents the first comprehensive phylogenetic analysis of Japanese Cyperaceae using *ndhF*. The *ndhF* analysis of suprageneric relationships in the family has shown better resolution than the *rbcL* analyses of Muasya *et al.* (1998, 2000). Our analyses support the tribal classification of Goetghebeur (1998), but the monophyly of subfamily *Cyperoideae* is not supported. Our data also show no congruence of the subfamilial classifications of Koyama (1961) and Bruhl (1995).

Mutations of 5.8S rDNA

Three base substitutions and 1-bp deletion (positions 128, 129, 136, and 168) support the monophyly of Cyperaceae (Table 2, Fig. 2). Starr *et al.* (1999) reported a 3-bp insertion (CAT) near the 3′ end of the 5.8S region in tribe *Cariceae* (position 155-157). Yano *et al.* (2004) found five types of 3-bp indels (CAC, CAT, CTC, CTT, and 3-bp deletion) in *Eleocharis*. In our analysis, a 3-bp indel (CAT) is found in many genera of Cyperaceae, and 3-bp deletion is found in 10 genera (Table 2). Our results indicate that a 3-bp insertion (CAT) occurred in the ancestor of Cyperaceae, and a 3-bp deletion occurred in four different evolutionary lineages (Fig. 2). Starr *et al.* (1999) and Yano *et al.* (2004) proposed that 3-bp indels are useful to delimit a clade within a genus or tribe. In our analyses, a 3-bp deletion supports the *Kyllinga* to *Actinoscirpus* and *Machaerina* clades. In addition, *Schoenus* is divided into two groups by 3-bp indels (CAT or 3-bp deletion). These results suggest that 3-bp indels are useful in obtaining a better understanding of intra- and inter- generic relationships in Cyperaceae.

Subfamily Mapanioideae

Goetghebeur (1998) recognized four subfamilies in Cyperaceae: *Mapanioideae*, *Sclerioideae*, *Caricoideae*, and *Cyperoideae* (Fig. 1). Subfamily *Mapanioideae* was also recognized by Koyama (1961). It is generally recognized that the *Mapanioideae* are the most primitive subfamily of Cyperaceae, based on floral morphology (Holttum 1948, Kern 1974). In the *ndhF* tree, subfamily *Mapanioideae* is placed as a sister to the rest of the Cyperaceae (Fig. 2). A similar position for subfamily *Mapanioideae* was found by Muasya *et al.* (1998, 2000) and Simpson *et al.* (2003). Our findings support the belief that subfamily *Mapanioideae* is the most primitive subfamily in Cyperaceae.

Subfamily Sclerioideae

Subfamilies *Sclerioideae* and *Caricoideae* have spicate spikelets composed of unisexual flowers. However, subfamily *Caricoideae* differs from subfamily *Sclerioideae* by having perigynia in the pistillate flowers (Koyama 1961, Goetghebeur 1998). Goetghebeur (1998) recognized four tribes *Bisboeckelereae*, *Cryptangieae*, *Sclerieae*, and *Trilepideae* in subfamily *Sclerioideae*. In the *ndhF* tree, tribes *Bisboeckelereae* and *Sclerieae* form a clade (99% LBP). Muasya *et al.* (1998, 2000) also reported tribe *Bisboeckelereae* to form a clade with tribe *Sclerieae* using *rbcL* analyses (93 or 99% BP: bootstrap probabilities).

Subfamilies Cyperoideae *and* Caricoideae

Goetghebeur (1998) merged seven tribes with spicate spikelets composed of bisexual flowers into subfamily *Cyperoideae*, and classified tribe *Cariceae*, which have unisexual flowers and a perig-

ynia, into subfamily *Caricoideae*. Several morphological analyses (Bentham 1883, Clarke 1908, Mattfeld 1936, Koyama 1961, Bruhl 1995) suggested that subfamily *Caricoideae* are closely related to tribe *Sclerieae*, which were treated as a member of subfamily *Sclerioideae* in Goetghebeur (1998). In the *rbcL* trees of Muasya *et al.* (1998, 2000), subfamily *Caricoideae* is not related to tribe *Sclerieae*, but is nested within the clade of subfamily *Cyperoideae* with low bootstrap support. Our *ndhF* tree strongly supports the monophyly of subfamily *Caricoideae* and its nesting within subfamily *Cyperoideae*. It also indicates that subfamily *Caricoideae* is related to tribe *Scirpeae* of subfamily *Cyperoideae* with strong support (100% LBP). Taxonomic revision is therefore needed for subfamilies *Cyperoideae* and *Caricoideae*.
Based on our findings, we propose that subfamily *Caricoideae* be treated as a member of subfamily *Cyperoideae*.

Phylogenetic relationship among tribes in the clade Cyperoideae

Goetghebeur (1998) classified seven tribes, *Abildgaardieae*, *Cypereae*, *Dulichieae*, *Eleocharideae*, *Fuireneae*, and *Scirpeae*, in subfamily *Cyperoideae* (Fig. 1). All tribes except *Schoeneae* form a monophyletic group with the *Cariceae* in subfamily *Caricoideae* in the *ndhF* tree, with two clades distinguished within this group: (1) *Dulichieae* to *Cariceae*, and (2) *Cypereae* to *Abildgaardieae* (Fig. 2). Tribes in the first clade are mainly distributed in the northern hemisphere; tribes in the second clade have a cosmopolitan distribution (Goetghebeur 1998). Our data show that these two clades diverged early in the evolution of the *Cypereae* to *Cariceae* clade.

The second clade comprises of two subclades, *Eleocharideae* to *Abildgaardieae* and *Cypereae* to *Fuireneae*. Svenson (1939) proposed that *Eleocharis* of tribe *Eleocharideae* is closely related to the *Fimbristylis* of tribe *Abildgaardieae* based on morphological characters. Recent morphological studies (Tucker 1987, Bruhl 1995, Goetghebeur 1998), however, proposed that the *Eleocharideae* are closely related to *Fuireneae*. Muasya *et al.* (1998, 2000), based on by *rbcL* analyses (< 50 or 54% BP), reported that tribes *Eleocharideae* and *Fuireneae* formed a clade. Roalson & Friar (2000) and Yano *et al.* (2004), however, reported tribes *Eleocharideae* and *Abildgaardieae* form a clade (52 or 86% BP) based on ITS analyses. In the *ndhF* tree, tribes *Eleocharideae* and *Abildgaardieae* form a clade (93% LBP). In the 5.8S rDNA, a single base substitution is shared in these two tribes (position 11). Our data support a shared more recent common ancestry of tribes *Eleocharideae* and *Abildgaardieae*, but not *Fuireneae*.

In the tribe *Schoeneae*, three clades were distinguished by *ndhF* analysis (Fig. 2). In addition, tribe *Schoeneae* was divided into two groups by a single base substitution in the 5.8S rDNA (position 135). Our data indicate that tribe Schoeneae is polyphyletic. Taxonomic revision is needed for tribe *Schoeneae*.

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References

Adachi, J. & M. Hasegawa. 1996. Computer science monographs 28. MORPHY version 2. 3. Programs for molecular phylogenetics based on maximum likelihood. Institute of Statistical Mathematics, Tokyo.

Bentham, G. 1883. Cyperaceae. *In*: Bentham, G. & J. D. Hooker (eds.), Genera Plantarum 3: 1037-1073. L. Reeve and Co., London.

Bohns, L. & R. G. Olmstead. 1997. Phylogenetic relationships in *Solanum* (Solanaceae) based on *ndhF* sequences. Syst. Bot. 22: 5-18.

Bruhl, J. J. 1995. Sedge genera of the world: relationships and a new classification of the Cyperaceae. Aust. Syst. Bot. 8: 125-305.

Clark, L. G., W. Zhang & J. F. Wendel. 1995. A phylogeny of the grass family (Poaceae) based on *ndhF* sequence data. Syst. Bot. 20: 436-460.

Clarke, C. B. 1908. New genera and species of Cyperaceae. Kew. Bull. Misc. Inform. Add. Ser. 8: 1-196.

Dallwitz, M. J., T. A. Paine & E. J. Zurcher. 1993. User's guide to the DELTA system. CSIRO, Melbourne.

Felsenstein, J. 1980-2001. PHYLIP (Phylogeny Inference Package) and manual, version 3.573c. Dept. Genetics, Univ. of Washington, Seattle.

——. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17: 368-376.

Fitch, W. M. 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. Syst. Zool. 20: 406-416.

Givnish, T. J., J. C. Pires, S. W. Graham, M. McPherson, L. M. Prince, T. B. Patterson, H. Rai, E. H. Roalson, T. M. Evans, W. J. Hahn, K. C. Millam, M. Molvray, P. J. Kores, H. O'Brien, J. C. Hall, W. J. Kress & K. J. Sytsma. 2002. A monocot-wide phylogeny based on the highly informative cpDNA gene *ndhF*: evidence for widespread concerted convergence. Direct Submission, Washington State University, Pullman.

Goetghebeur, P. 1998. Cyperaceae. *In*: Kubitzki, K. (ed.), The families and genera of vascular plants 4. Flowering plants, monocotyledons: Alismatanae and Commelinanae (except Gramineae): 141-190. Springer, Berlin.

Hasegawa, M., H. Kishino & T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22: 160-174.

Hiratsuka, J., H. Shimada, R. Whittier, T. Ishibashi, M. Sakamoto, M. Mori, C. Kondo, Y. Honji, C. R. Sun, B. Y. Meng, Y. Q. Li, A. Kanno, Y. Nishizawa, A. Hirai, K. Shinozaki & M. Sugiura. 1989. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. 217: 185-194.

Holttum, R. E. 1948. The spikelet in Cyperaceae. Bot. Rev. 14: 525-541.

Hsiao, C., N. J. Chatterton, K. H. Asay & K. B. Jensen. 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. Genome 37: 112-120.

Kern, J. H. 1974. Cyperaceae 1. *In*: C. G. G. J. van Steenis (ed.), Flora Malesiana, ser. 1, 7(3): 435-753. Noordhoff, Leyden.

Koyama, T. 1961. Classification of the family Cyperaceae 1. J. Fac. Sci. Univ. Tokyo sect. 3, 8: 37-148.

Kumar, S., K. Tamura, I. B. Jakobsen & M. Nei. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244-1245.

Mattfeld, J. 1936. Cyperaceae. *In*: Engler, A. and L. Diels (eds.), Syllabus der Pflanzenfamilien 11: 142-143. Gebruder Borntraeger, Berlin.

Muasya, A. M., J. J. Bruhl, D. A. Simpson, A. Culham & M. W. Chase. 2000. Suprageneric phylogeny of Cyperaceae: a combined analysis. *In*: Wilson, K. L. & D. A. Morrison (eds.), Monocots: Systematics and Evolution: 593-601. CSIRO, Melbourne.

——, D. A. Simpson, M. W. Chase & A. Culham. 1998. An assessment of suprageneric phylogeny in Cyperaceae using *rbcL* DNA sequences. Plant Syst. Evol. 211: 257-271.

Nixon, K. C. 1999. The Parsimony Ratchet, a new method for rapid parsimony analysis. Cladistics 15: 407-414.

Ohwi, J. 1944. Cyperaceae Japonicae, Pt. 2. A synopsis of the *Rhynchosporoideae* and *Scirpoideae* of Japan, including the Kuriles, Saghalin, Korea, and Formosa. Mem. Coll. Sci. Kyoto Imp. Univ. B, 18: 1-182.

Olmstead, R. G. & J. A. Sweere. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in Solanaceae. Syst. Bot. 43: 467-481.

Olsen, G. J., H. Matsuda, R. Hagstrom & R. Overbeek. 1994. FastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. 10: 41-48.

Partridge, T. R., M. J. Dallwitz & L. Watson. 1993. Delta primer. A general system for processing taxonomic descriptions. CSIRO, Melbourne.

Roalson, E. H. & E. A. Friar. 2000. Infrageneric classification of *Eleocharis* (Cyperaceae) revisited: evidence from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Syst. Bot. 25: 323-336.

Shimodaira, H. & M. Hasegawa. 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246-1247.

Simpson, D. A., C. A. Furness, T. R. Hodkinson, A. M. Muasya & M. W. Chase. 2003. Phylogenetic relationships in Cyperaceae subfamily *Mapanioideae* inferred from pollen and plastid DNA sequence data. Am. J. Bot. 90: 1071-1086.

Starr, J. R., R. J. Bayer & B. A. Ford. 1999. The phylogenetic position of *Carex* section *Phyllostachys* and ITS implication for phylogeny and subgeneric circumscription in *Carex* (Cyperaceae). Amer. J. Bot. 86: 563-577.

Svenson, H. K. 1939. Monographic studies in the genus Eleocharis. Rhodora 41: 3-19; 95-104.

Swofford, D. L. 2003. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Takaiwa, F., K. Oono & M. Sugiura. 1985. Nucleotide sequence of the 17S-25S spacer region from rice rDNA. Pl. Mol. Biol. 4: 355-364.

Terry, R. G., G. K. Brown & R. G. Olmstead. 1997. Phylogenetic relationships in subfamily *Tillandsioideae* (Bromeliaceae) using *ndhF* sequences. Syst. Bot. 22: 333-346.

Thompson, J. D., D. G. Higgins & T. J. Gibson. 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22: 4673-4680.

Tucker, G. C. 1987. The genera of Cyperaceae in the southeastern United States. J. Arnold Arbor. 68: 361-445.

Tsubota, H., Y. Ageno, B. Estébanez, T. Yamaguchi & H. Dehuchi. 2003. Molecular phylogeny of the Grimmiales (Musci) based on chloroplast *rbcL* sequences. Hikobia 14: 55-70.

——, T. Arikawa, H. Akiyama, E. De Luna, D. Gonzárez, M. Higuchi & H. Deguchi. 2002. Molecular phylogeny of hypnobryalean mosses as inferred from a large-scale dataset of chloroplast *rbcL*, with special reference to the Hypnaceae and possibly related families. Hikobia 14: 55-70.

White, T. J., T. Bruns, S. Lee & J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M. A., D. H. Gelfand, J. J. Sninsky & T. J. White (eds.), PCR protocols, a guide to methods and applications: 315-322. Academic Press, New York.

Yano, O. & T. Hoshino. 2005. Molecular phylogeny and chromosomal evolution of Japanese *Schoenoplectus* (Cyperaceae), based on ITS and ETS 1f sequences. Acta Phytotax. Geobot. 56: 183-195.

——, T. Katsuyama, H. Tsubota & T. Hoshino. 2004. Molecular phylogeny of Japanese *Eleocharis* (Cyperaceae) based on ITS sequence data, and chromosomal evolution. J. Plant Res. 117: 409-419.

Yen, A. C. & R. G. Olmstead. 2000. Molecular systematics of Cyperaceae tribe *Cariceae* based on two chloroplast DNA regions: *ndhF* and *trnL* intron-intergenic spacer. Syst. Bot. 25: 479-494.

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